INTRACELLULAR DELIVERY OF PROTEINS AND NUCLEIC ACIDS TO MAMMALIAN CELLS WITH INVAPLEX ISOLATED FROM *SHIGELLA FLEXNERI*

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ABSTRACT

The invasion plasmid antigens (Ipa) of *Shigella* spp, are effector molecules necessary for the invasion of colonic epithelial cells. Recently, we have isolated an invasin protein-LPS complex from intact, virulent *Shigella* (Invaplex). *In vitro* observations suggest that Invaplex interacts with host-cell membranes, is internalized, and is released into the cytoplasm via a process similar to the activity expressed by virulent shigellae. It was therefore hypothesized that if heterologous molecules (DNA or protein) were present at the time of Invaplex-induced endocytosis, the heterologous molecule would also be taken up by the host cell. Using plasmid DNA encoding either GFP, β-galactosidase or scrub typhus Sta56 protein, Invaplex was found to induce the uptake of plasmids into host cells resulting in intracellular expression. Invaplex also mediated the transport of purified proteins (GFP, β-galactosidase) across mammalian cell membranes while retaining protein functionality. *In vivo* experiments demonstrated that Invaplex enhanced the immune response to co-delivered proteins and DNA.

1. INTRODUCTION

The pathogenesis of *Shigella* spp. (the causative agent of bacillary dysentery) is attributed to the organism's ability to invade, replicate intracellularly, and spread intercellularly within the colonic epithelium. Upon contact or attachment to host cells, the *Shigella* invasins (IpaB and IpaC) (Buysse et al. 1987) induce a phagocytic event resulting in engulfment and internalization of the bacterium by the host cell (Menard et al. 1993). We have isolated an invasin protein-LPS complex (Invaplex) from virulent *Shigella* cells (Turbyfill et al. 2000) that maintains the key antigenic components of *Shigella* in a functional and immunogenic state. In mouse and guinea pig models, *Shigella* Invaplex administered intranasally is immunogenic and protective against *Shigella* challenge (Turbyfill et al. 2000).

The immunogenicity of Invaplex (IVP) is thought to be related to its ability to bind to epithelial cell surfaces, followed by endocytosis (Kaminski et al. 2004) and eventual presentation to the mucosal immune system. Once internalized, Invaplex traffics via endosomes to the Golgi apparatus and eventually into the host cell cytosol (Kaminski et al. 2004). The ability to bind to a eukaryotic cell surface and induce an endocytic event indicates that the Invaplex maintains an active, native virulence structure similar to that found on the surface of invasive *Shigella*. Our recent research has been directed at utilization of the native properties of Invaplex, not only as a *Shigella* vaccine, but as a biological system capable of efficient delivery of proteins and nucleic acids across eukaryotic cell membranes. Such delivery, followed by presentation to the immune system would enhance the immune response to the co-delivered protein antigens and/or nucleic acids from vaccine candidates. The studies below describe the ability of Invaplex to stimulate the transport of proteins and DNA across eukaryotic membranes in an Ipa protein-dependent manner and enhance the immunogenicity of both plasmid DNA-based and protein subunit-based mucosal vaccines.

2. MATERIALS AND METHODS

Invaplex-Mediated Transport of Purified Green Fluorescent Protein or Beta-Galactosidase Across Mammalian Cell Membranes. The ability of Invaplex to mediate the transport of the green fluorescent protein (GFP) or beta-galactosidase (β-gal) protein across plasma membranes was investigated by incubating semi-confluent BHK-21 monolayers with *S. flexneri* 2a IVP-24 or 50 (50 to 350 μg/ml) mixed with either purified GFP (5μg/ml) or purified β-gal protein (50 units/ml). Controls for the assay included monolayers incubated with either minimal essential mediu (MEM) alone, GFP or β-gal diluted in MEM. All monolayers were incubated overnight at 37°C with the Invaplex-protein mixtures. After washing, cells in the monolayer were scored as GFP-positive or GFP-negative based on the presence or absence of cell-associated green fluorescence, respectively. Intracellular β-gal was detected with X-gal staining buffer (Genetic Therapy Systems). β-gal-positive cells had a blue-stained cytoplasm when viewed by light microscopy.

Transfection of mammalian cells with plasmid DNA encoding either GFP, scrub typhus antigen Sta56 or β-gal **using *Shigella* Invaplex as the transfection**

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mediation reagent. The ability of *Shigella* Invaplex to mediate the transfection of cells with plasmid DNA was assessed by incubating BHK-21 monolayers with plasmid DNA (0.5 µg) encoding either GFP (pEGFP-N1; Gene Therapy Systems), β-gal (gWiz β-galactosidase plasmid, Gene Therapy Systems) or the scrub typhus *sta*56 gene (Oaks et al. 1987) (pVR1012_*sta*56) combined with *S. flexneri* 2a Invaplex. Plasmid gene expression was under the control of a eukaryotic cytomegalovirus (CMV) promoter. Monolayers incubated with MEM alone, 0.5 µg of pEGFP-N1 or gWiz β-gal diluted in MEM served as the negative controls for the assay. After an overnight incubation, cells were washed and stained for GFP or β-gal activity as indicated above. Intracellular expression of Sta56 was detected using indirect immunofluorescent microscopy wtih the K13F88A monoclonal antibody directed to Sta56 (Stover et al. 1990)

Invaplex-Sta56 DNA Immunogenicity Study. The ability of *Shigella* Invaplex to enhance the immunogenicity of a plasmid DNA-based vaccine was evaluated in mice. Groups of female Balb/cByJ mice (10 mice/grp) were intranasally immunized on day 0, 14, and 28 with plasmid DNA containing the scrub typhus *sta*56 gene (Oaks et al. 1987) from the Karp strain of *Orienta tsutsugamushi* linked to a CMV promoter (pVR1012_*sta*56). Mice were intranasally immunized with pVR1012_*sta*56 alone or combined with *S. flexneri* 2a IVP-50 (15 µg). Controls for the study included groups of mice intranasally immunized with either saline, *S. flexneri* 2a IVP-50 (15 µg) or the empty expression vector (pVR1012) (100 µg) combined with *S. flexneri* 2a IVP-50 (15 µg). Five mice per group (Subgroup "A") were euthanized two weeks after the third DNA immunization when blood and lymphoid cells were harvested. The remaining five mice per group (Subgroup "B") were boosted on day 56 with an intranasal immunization of purified recombinant Sta56 protein (15 µg) combined with *S. flexneri* 2a IVP-50 (5 µg). Groups of mice receiving the Invaplex-Sta56 protein booster immunization were euthanized two weeks after the booster immunization (day 70) when blood and lymphoid cells were harvested.

Invaplex-FlaA Immunogenicity Study. Groups of mice (5 mice/grp) were intranasally immunized on day 0, 14, and 28 with the recombinant *Campylobacter coli* flagellin A (FlaA) protein (Lee et al. 1999) delivered alone (5 or 50 µg) or combined with *Shigella* Invaplex. Control groups were immunized with saline or *Shigella* Invaplex. Serum was collected on day 0, 28, 35, and 42. Mucosal washes were collected from the lung and intestine on day 42.

Antigen-specific ELISAs and proliferation assays. Antigen-specific antibody responses were assessed in the mucosal washes and serum samples by an enzyme linked immunosorbant assay (ELISA) as previously described (Turbyfill et al. 2000). Coating concentrations of the various antigens plated at 50 µl/well were: *S. flexneri* 2a IVP-50 at 1 µg/ml, FlaA at 0.6 µg/ml and the Sta56 protein at 3 µg/ml. Splenocytes were evaluated for antigen-specific proliferation by culturing 1 x 10^5 lymphoid cells with Sta56, *Shigella* Invaplex, or the mitogen concanavalin A (Con A). Negative controls included immune cells incubated with medium alone to control for normal proliferation and cells from naive mice stimulated with antigen to control for non-specific proliferation.

3. RESULTS

Invaplex-mediated transport of heterologous proteins and nucleic acids. The ability of Invaplex to transport heterologous proteins across mammalian plasma membranes was assessed using two proteins with reporter functions. BHK-21 cells incubated overnight with purified GFP (5 µg/ml) mixed with either Invaplex-24 (75 µg/ml) or Invaplex-50 (35 µg/ml) resulted in the intracellular transport of GFP into 47% and 57% of cells, respectively, compared with < 1% in cells treated with GFP mixed with culture media (Table 1). Incubation of cells with the same amount of GFP and higher concentrations of Invaplex-24 and Invaplex-50 did not result in a higher percentage of GFP positive cells.

Table 1. Invaplex-mediated transport of heterologous proteins.

Treatment	Amount (µg/ml)	Mean Percent GFP Positive	Mean Percent β-gal Positive
MEM	N/A	< 1%	< 1%
MEM	N/A	2%	< 1%
S. flexneri 2a IVP-24	75	47%	34%
S. flexneri 2a IVP-24	165	34%	28%
S. flexneri 2a IVP-24	330	17%	9%
S. flexneri 2a IVP-50	35	57%	47%
S. flexneri 2a IVP-50	70	43%	22%
S. flexneri 2a IVP-50	140	12%	8%

BHK-21 cells incubated overnight with β-gal (50 units/ml) mixed with either Invaplex-24 (75 µg/ml) or Invaplex-50 (35 µg/ml) resulted in the intracellular transport of β-gal in 34% and 47% of cells, respectively, compared with < 3% in cells incubated with purified β-

gal protein co-mixed with culture media. Incubation of cells with the same amount of β-gal and higher concentrations of Invaplex-24 and Invaplex-50 did not result in a higher percentage of β-gal-positive cells.

Separate BHK-21 monolayers were incubated with plasmid DNA expressing either GFP (pEGFP; 0.5 µg), the scrub typhus antigen Sta56 (pVR1012_*sta*56; 0.5 µg) or β-gal (gWIZ_Bgal; 0.5µg) mixed with either culture media (MEM) (negative control), a known transfection reagent (GenePorter), Invaplex-24 (100 or 500 µg/ml) or Invaplex-50 (100 or 500 µg/ml). Greater than 200 cells per monolayer were then scored as being GFP-positive (or β-gal-positive) or negative (Table 2).

Table 2. Invaplex-mediated transport of plasmid DNA constructs.

Transfection Reagent	Amount (µg/ml)	Percent GFP positive	Percent β-gal positive	Percent Sta56 positive
MEM	N/A	< 1%	< 1%	< 1%
GenePorter	10 µl	65%	60%	N/D
S. flexneri 2a IVP-24	100	16%	22%	24%
S. flexneri 2a IVP-24	500	20%	29%	N/D
S. flexneri 2a IVP-50	100	19%	14%	19%
S. flexneri 2a IVP-50	500	22%	15%	N/D

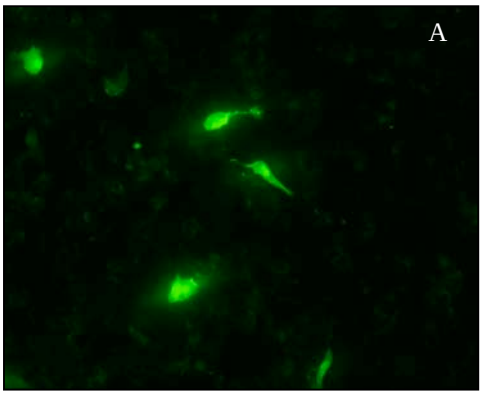
Abbreviations: N/D = not done.

Expression of GFP was detected in 16% and 19% of cells incubated with pEGFP mixed with either Invaplex-24 or Invaplex-50, respectively, (see Fig. 1A) whereas incubation with pEGFP alone did not result in the intracellular expression of GFP (Table 2).

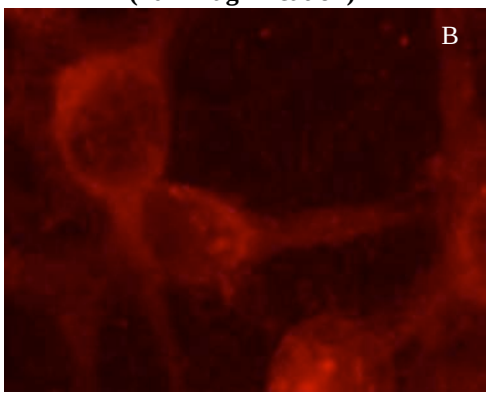
Expression of the *sta*56 gene was detected in 24% and 19% of the BHK-21 cells incubated with pVR1012_*sta*56 co-mixed with Invaplex (Fig. 1B) whereas expression of *sta*56 could not be detected after incubation with pVR1012_*sta*56 alone. Expression of β-gal was detected in 22% and 14% of cells incubated with gWIZ_β-gal and either Invaplex-24 or Invaplex-50, respectively, (Fig. 1C) whereas incubation of cells with gWIZ_β-gal alone did not result in the intracellular expression of β-gal. Incubation of cells with the same amount of plasmid DNA and higher concentrations of Invaplex-24 and Invaplex-50 resulted in nominal increases in the transfection efficiency

Invaplex as an immunological adjuvant for the delivery of DNA-based vaccines.

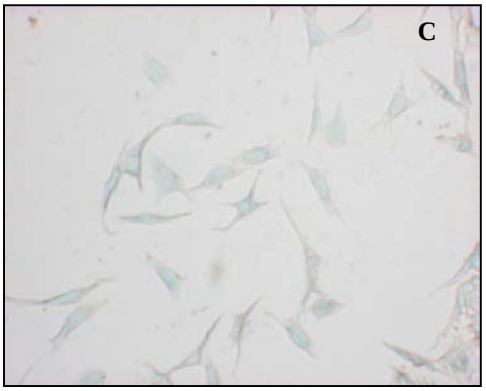
Mice intranasally immunized with Invaplex combined with either proteins or pDNA seroconvert to the antigen



Invaplex + pEGFP (10X magnification)



Invaplex + pVR1012_*sta*56 (60X magnification)



Invaplex + gWIZ_BGal (10X magnification)

Figure 1. Intracellular expression of genes encoded on plasmid DNA transported into mammalian host cells by *Shigella* Invaplex. BHK-21 cells were incubated with co-mixtures of *Shigella* Invaplex and plasmid DNA encoding either, (A) *gfp*, (B) the *sta*56 gene from *Orientia tsutsugamushi*, or (C) *lacZ*. Expression of the *lacZ* gene was determined with a colorimetric substrate, GFP with fluorescent microscopy, and *sta*56 utilizing indirect immunofluorescence.

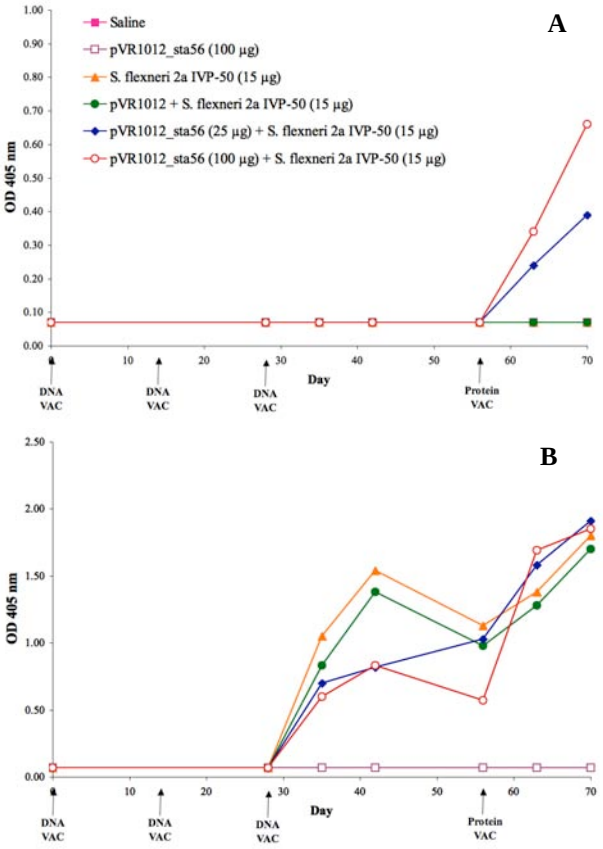


Figure 2. Sta56 and Invaplex-specific serum IgG responses after intranasal immunization utilizing an Invaplex-DNA prime-Invaplex-protein boost vaccine regimen.
Groups of mice were intranasally immunized on day 0, 14, and 28 with plasmid DNA encoding the *sta*56 gene from *O. tsutsugamushi* either alone or combined with *S. flexneri* IVP-50. Control groups were immunized with saline, pVR1012, or *S. flexneri* IVP-50. Blood was taken from all animals before immunization on day 0, and 28, and on day 35, 42, 56, 63, and 70. The Sta56-specific (Figure 2A) and *S. flexneri* IVP-50-specific (Figure 2B) serum IgG responses were determined by ELISA. OD_{405} values represent the mean OD_{405} at a 1:180 dilution of sample after a 60 minute incubation with substrate for each group of mice (n = 5/grp).

delivered with the Invaplex. Groups of mice were intranasally immunized with pDNA encoding the scrub typhus *sta*56 gene (pVR1012_*sta*56) either alone or co-administered with Invaplex and boosted with purified Sta56 protein plus Invaplex. Immunization with Invaplex and pVR1012_*sta*56 resulted in Sta56-specific serum IgG and Invaplex-specific serum IgG responses measured 1 and 2 weeks after the booster immunization (Figure 2A). Invaplex-specific serum IgG responses were present in all groups receiving Invaplex alone or combined with pDNA constructs after three intranasal immunizations and were subsequently boosted after the Invaplex-Sta56 immunization on day 56 (Figure 2B). Sta56-specific (Table 3) and Invaplex-specific (data not shown) cell-mediated immune responses were detected in splenocytes from the group of mice immunized with pVR1012_*sta*56 combined with Invaplex after *in vitro* stimulation. Intranasal immunization with pVR1012_*sta*56 alone did not result in the induction of a detectable Sta56-specific serum IgG response or cell-mediated immunity, demonstrating that Invaplex was an effective mucosal adjuvant for the delivery of plasmid DNA-based vaccines while retaining the ability to elicit *Shigella*-specific immunity.

Table 3. Sta56-specific proliferation in splenocytes after intranasal immunization with plasmid DNA encoding the *sta*56 gene

Vaccine Group	Sta56-specific Proliferation (Mean Stimulation Index)	
	2 weeks Post 3rd DNA Vaccine	2 weeks Post Protein Boost
Saline	1.46	1.22
pVR1012_*sta*56	1.26	1.14
S. flexneri 2a IVP-50	1.32	1.28
pVR1012 + *S. flexneri* 2a IVP-50	1.12	1.38
pVR1012_*sta*56 (25 µg) + *S. flexneri* 2a IVP-50	7.04*	2.34
pVR1012_*sta*56 (100 µg) + *S. flexneri* 2a IVP-50	5.70*	3.40*

Table 3 legend. Sta56-specific proliferative responses were measured in splenocytes stimulated *in vitro* for 5 days with Sta56 using a colorimetric cell proliferation assay. The mean (n=5 mice/grp) stimulation index (SI) was determined by dividing the mean OD calculated for triplicate wells containing cells stimulated with Sta56 protein by the mean OD measured after incubation with culture media alone. * indicate values significantly greater ($p < 0.05$; unpaired t test) than the SI values calculated for the group of mice immunized with pVR1012_*sta56* alone.

Invaplex as an immunological adjuvant for the delivery of protein subunit vaccines. The ability of Invaplex to enhance the immunogenicity of a protein subunit vaccine was also investigated. The serum IgG and mucosal IgA antibody responses directed to the FlaA antigen were measured by ELISA (Figure 3). Intranasal

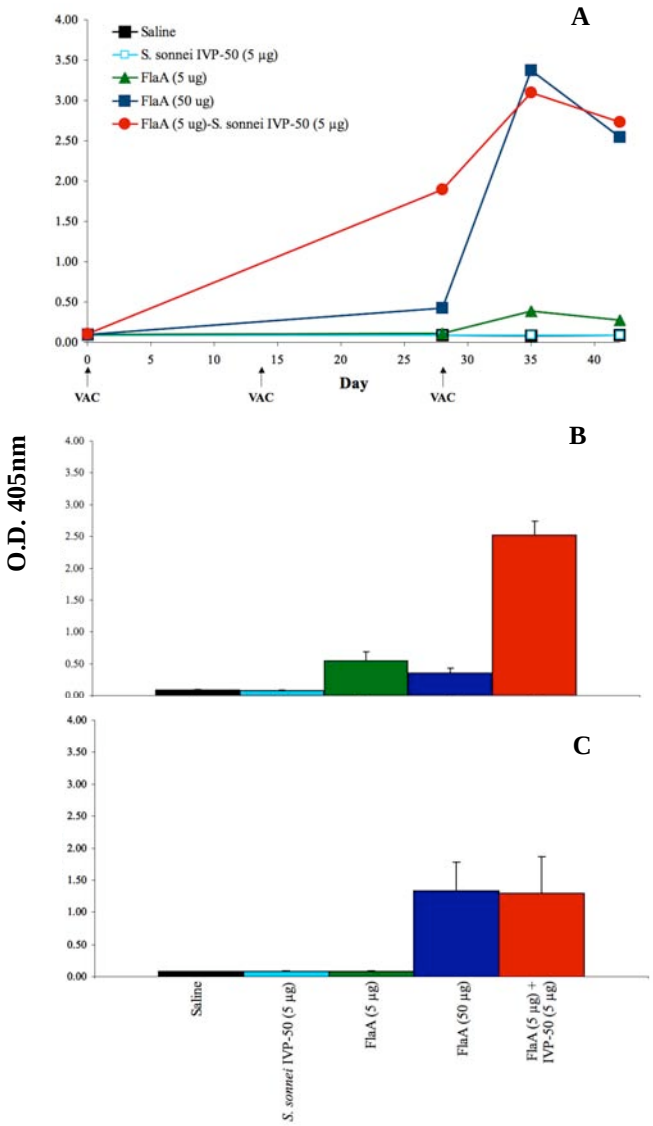


Figure 3. FlaA-specific antibody responses in murine sera, lung and intestinal washes after intranasal immunization with FlaA alone or FlaA delivered with *Shigella* Invaplex.
Groups of mice were intranasally immunized on day 0, 14, and 28 with FlaA alone or FlaA co-administered with *Shigella* Invaplex (IVP-50). Control groups were immunized with saline and *Shigella* Invaplex alone. FlaA-specific serum IgG was measured by ELISA in samples collected on day 0, 28, 35, and 42 (A). Mucosal washes collected on day 42 from the intestine (B), and the lung (C) were assayed for FlaA-specific IgA responses. Error bars represent the standard error of the mean of five mice/group.

immunization with FlaA (5 µg) elicited minimal FlaA-specific serum IgG, detectable only after three immunizations. In contrast, animals immunized with FlaA (5 µg) combined with *Shigella* Invaplex mounted a robust FlaA-specific serum IgG response after two immunizations indicating that formulation with Invaplex decreased the amount of immunizations and time required to mount a FlaA-specific immune response. Moreover, the FlaA-specific serum IgG response elicited after three immunizations with Invaplex-FlaA (5 µg) were comparable to those induced with FlaA (50 µg) and significantly higher (p = 0.02) than the response induced with FlaA (5 µg). Protection against mucosal pathogens may require the generation of antigen-specific mucosal antibody responses. Intranasal immunization with Invaplex-FlaA (5 µg) induced FlaA-specific IgA responses in mucosal compartments located both proximal (lung) and distal (intestine) to the immunization site (nasal) indicating that Invaplex-adjuvanted vaccines are capable of stimulating the common mucosal immune system **(**Mestecky 1987**)**. Immunization with FlaA (5 µg) alone did not induce detectable FlaA-specific IgA responses in either mucosal compartment.

4. DISCUSSION

The *Shigella* invasin complex (Invaplex) is a macromolecular complex isolated from water extracts of virulent *Shigella* cultures using ion-exchange FPLC with NaCl step gradients. Invaplex contains LPS and the plasmid-encoded proteins, IpaB and IpaC. Previous work has established Invaplex as a potent mucosal immunogen, capable of inducing protective, *Shigella*-specific immune responses as demonstrated in both mice and guinea pigs (Turbyfill, 2000). Invaplex is currently being evaluated in Phase 1 clinical trials as a vaccine to prevent shigellosis in deployed military troops.

In the current study, it was demonstrated that *Shigella* Invaplex mediated the transport of purified proteins (GFP and β-gal) across plasma membranes of mammalian cells. Purified GFP was transported across plasma membranes in a comparable percentage of cells with equivalent concentrations of Invaplex-24 and Invaplex-50 suggesting that both Invaplex preparations are equally capable of transporting proteins across mammalian plasma membranes. In addition, *Shigella* Invaplex mediated the uptake of plasmid DNA encoding GFP, Sta56, or β-gal resulting in the intracellular expression of heterologous proteins. These studies demonstrate that Invaplex stimulates the uptake of co-administered proteins and nucleic acids indicating that Invaplex may be useful for delivering prophylactic or therapeutic molecules across eukaryotic cell membranes.

The lack of cytotoxicity and transfection capacity of Invaplex lead to the hypothesis that Invaplex may be capable of functioning as a mucosal adjuvant for the delivery of DNA-based vaccine constructs. Using plasmid DNA encoding the gene for the scrub typhus antigen Sta56, it was demonstrated that *Shigella* Invaplex enhanced the cell-mediated immunogenicity of the DNA vaccines. Moreover, following a Sta56 protein-Invaplex booster immunization, Sta56-specific humoral immunity was detectable, indicating the DNA-Invaplex prime/protein-Invaplex booster vaccine regimen was capable of stimulating both cell-mediated and antibody responses. In other studies, the ability of Invaplex to enhance the immunogenicity of a protein subunit vaccine was demonstrated using the *Campylobacter* FlaA protein. Intranasal immunization with FlaA combined with Invaplex induced FlaA-specific antibody responses that were of higher magnitude than those induced with comparable amounts of FlaA delivered alone, and similar in magnitude to those induced with ten times more FlaA antigen. Furthermore, intranasal immunization with Invaplex-FlaA combination vaccines resulted in the induction of FlaA-specific mucosal antibody responses in compartments located both proximal (lung) and distal (intestine) to the site of immunization (intranasal). While enhancing the immunogenicity of the FlaA protein, Invaplex-specific immune responses were retained, which provides evidence that Invaplex-FlaA combination vaccines may be capable of inducing immunity to two enteric pathogens, potentially providing protection to deployed military troops against both pathogens with one vaccine. The adjuvant-like properties of Invaplex may be useful for delivery of protein subunit and DNA-based vaccines from biowarfare agents, diarrheal agents and respiratory agents that are targeted in the Army's vaccine development program.

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REFERENCES

Buysse, J. M., C. K. Stover, E. V. Oaks, M. Venkatesan, and D. J. Kopecko, 1987: Molecular cloning of invasion plasmid antigen (ipa) genes from *Shigella flexneri*: analysis of ipa gene products and genetic mapping. *J Bacteriol*, **169,** 2561-9.

Kaminski, R. W., K. R. Turbyfill, and E. V. Oaks, 2004: Internalization of Invaplex Isolated from *Shigella* flexneri and co-localization with intracellular organelles. *General Meeting of the American Society for Microbiology*, New Orleans, LA.

Lee, L. H., E. Burg, 3rd, S. Baqar, A. L. Bourgeois, D. H. Burr, C. P. Ewing, T. J. Trust, and P. Guerry, 1999: Evaluation of a truncated recombinant flagellin subunit vaccine against *Campylobacter jejuni. Infect Immun*, **67,** 5799-805.

Menard, R., P. J. Sansonetti, and C. Parsot, 1993: Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J Bacteriol*, **175,** 5899-906.

Mestecky, J., 1987: The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J Clin Immunol*, **7,** 265-76.

Oaks, E. V., C. K. Stover, and R. M. Rice, 1987: Molecular cloning and expression of *Rickettsia* tsutsugamushi genes for two major protein antigens in Escherichia coli. *Infect Immun*, **55,** 1156-62.

Stover, C. K., D. P. Marana, J. M. Carter, B. A. Roe, E. Mardis, and E. V. Oaks, 1990: The 56-kilodalton major protein antigen of *Rickettsia tsutsugamushi:* molecular cloning and sequence analysis of the sta56 gene and precise identification of a strain-specific epitope. *Infect Immun*, **58,** 2076-84.

Turbyfill, K. R., A. B. Hartman, and E. V. Oaks, 2000: Isolation and characterization of a *Shigella flexneri* invasin complex subunit vaccine. *Infect Immun*, **68,** 6624-32.